

Interrelationship of Temperature, Flower Development and Biological Control of Fire Blight

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Abstract

BlightBan (a.i., *Pseudomonas fluorescens* strain A506) has been available in recent years and other biological agents (e.g., *Pantoea agglomerans* strains C9-1 and E325) are being developed for fire blight control. Advances will partly depend on an understanding of interrelationships involving environment, flower development and senescence, and microorganisms. Laboratory experiments were performed with detached blossoms of crab apple (*Malus* sp. 'Manchurian'). When inoculated stigmas were held at various temperatures, strain A506 failed to grow at the upper temperature range of *Erwinia amylovora*, but strains C9-1 and E325 had ranges extending beyond that of the pathogen. When flowers were subject to various temperatures and incubation periods before inoculation, the duration of stigma conduciveness to bacterial multiplication decreased as temperature increased, and it was shorter when flowers were pollinated versus non-pollinated. These interactions were confirmed with mature apple trees (*Malus x domestica* Borkh. 'Gala') surrounded by plastic enclosures with heating and cooling to maintain different temperature ranges. In trials in 2000 and 2001, temperatures during bloom averaged 13.4° and 14.1° C at the low range and 21.5° and 21.9°C at the high range, respectively. The longest period stigmas retained the capacity to support bacterial growth when inoculated was about 14 days, occurring with non-pollinated flowers at low temperatures. The shortest period was 3 or 4 days, occurring with pollinated flowers at high temperatures. Results were similar with different bacteria. However, strain A506 failed to multiply at late stages of stigma senescence, which did allow growth of the pathogen and strain E325. On crab apple flowers, antagonist strains differed in their capacity to reduce pathogen populations and these differences were consistent during the period when stigmas were conducive to pathogen growth.

INTRODUCTION

Biological control is still a relatively new tool for managing fire blight of apple and pear caused by *Erwinia amylovora*. The product **BlightBan** (a.i., *Pseudomonas fluorescens* strain A506) has been available in recent years and other biological agents (e.g., *Pantoea agglomerans* strains C9-1 and E325) are currently being developed commercially for this disease. Experience with these beneficial bacteria indicates an inconsistency possibly related to environment and the plant host. Improvements in efficacy will partly depend on an understanding of these factors. The flower part of greatest interest is the stigma, where *E. amylovora* can become established even under dry western conditions. It is known that temperature affects the duration that flower stigmas are receptive to pollen and that stigma senescence is accelerated after pollination (Soltesz et al., 1996). Also, a relationship between stigma age and colonization by *E. amylovora* has been reported (Gouk and Thomson, 1999). Information is lacking, however, regarding the relationship of these factors to biological control. This study examines the interrelationship of temperature, pollination, stigma age, and microbial colonization and interactions.

MATERIALS AND METHODS

Plant Material

Laboratory experiments were performed with detached blossoms of 'Manchurian' crab apple trees (1.6 cm minimum stem diameter) received from a local nursery and induced to bloom in a greenhouse as described previously (Pusey, 1997). Newly opened flowers with non-dehiscent anthers were collected and maintained by submerging the cut end of the pedicle in 10% sucrose contained in a 2-ml vial. Vials with flowers were supported in 4-liter chambers and, unless otherwise indicated, relative humidity (RH) was established at 90% by flooding the bottom of the containers with 1 L of a glycerol solution (Johnson, 1940).

Field experiments were performed in 2000 and 2001 with 'Gala' apple trees in an experimental block at Columbia View near Wenatchee, Washington. Trees were 6-years old in the first year.

Bacterial Strains and Inoculation

Bacterial strains used were *E. amylovora* strain Ea153 (Johnson et al., 1993) and antagonists *P. fluorescens* strain A506 (Lindow et al., 1996) and *P. agglomerans* strains E325 (Pusey, 1997 and 1999) and C9-1 (Ishimaru, 1984; Johnson et al., 1993). Strain Ea153 was previously marked with nalidixic acid resistance and antagonist strains were marked with rifampicin resistance.

Bacteria were cultured on nutrient yeast dextrose agar (NYDA; nutrient broth, 8 g; yeast, 5 g; dextrose, 5 g; agar, 15 g; and deionized water, 1 liter) for 24 h at 24° C, and inoculum suspensions were prepared in 10 mM potassium phosphate buffer (pH 7.0) and 0.03% Tween-20. Flowers were inoculated with micropipette by applying about 0.1 ul of the suspension (10^6 , 10^7 or 10^8 cfu/ml) to the stigmatic surfaces of each flower.

Population size of bacterial strains on flower stigmas was determined by placing the stigmas, along with portions of the supporting styles, in sterile microcentrifuge tubes containing 1 ml of sterile buffer (10 mM potassium phosphate, pH 7.0). Tubes were vortexed briefly and placed in a sonication bath for 60 s. Samples were again vortexed, and serial dilutions were spread on CCT medium (Ishimaru and Klos, 1984) amended with nalidixic acid (100 µg/ml) for detection of strain Ea153 or on NYDA amended with 25 ppm rifampicin and 50 ppm cycloheximide for detection of strains E325, C9-1 and A506. Plates were incubated at 24°C.

Fluctuating versus Constant Diurnal Temperatures

To study the effects of fluctuating versus constant diurnal temperatures on microbial growth on the stigma, detached crab apple flowers were inoculated with Ea153 by applying a suspension of 10^6 cfu/ml to the stigmatic surfaces of each flower. Flowers were supported in open containers placed in four different controlled environment chambers, each programmed for a different diurnal temperature range (14° C constant, 10 to 18° C, 7 to 21° C or 4 to 24° C). In each chamber, the daily average temperature was 14° C. All chambers were set at a constant of 90% relative humidity. Ten flowers were sampled after 0, 24, 48, and 72 h to determine bacterial population size. Data from two trials were pooled.

Temperature Range and Optimum for Microbial Growth

To determine the temperature range and optimum for growth of bacterial strains on flower stigmas, stigmas of detached crab apple flowers were inoculated with strain Ea153, E325, C9-1 or A506 using a suspension of 10^8 cfu/ml. The estimated starting population size on each flower was about 10^4 cfu. Flowers were incubated for 24 h at temperatures ranging from 4 to 40° C and at increments of 4° C before determining bacterial population size. Five flowers were used per strain per temperature. Data from three trials were pooled.

Temperature, Pollination and Stigma Age in Laboratory

The effect of temperature, pollination and flower stigma age on bacterial growth on stigmas was studied. Crab apple flowers were collected, anthers removed with micro scissors, then half of the flowers were pollinated by applying a mixture of 'Rome' and 'Red Delicious' apple pollen to stigmas with a paint brush and the other half were left unpollinated. Flowers were held for 0, 4 or 8 days at 4, 14 or 24° C prior to inoculation with strain Ea153, E325 or A506 using a suspension of 10^8 cfu/ml. (Flowers were collected on different dates, but all flowers were inoculated on the same date.) After inoculation, flowers were incubated for 24 h at 24° C. Five pollinated and five non-pollinated flowers were used per stigma age and temperature. Data from two trials were pooled.

Temperature, Pollination and Stigma Age in Field

The above relationships were also investigated using 'Gala' apple trees. Each tree was enclosed in an aluminum frame, 3 m wide x 2.4 m long x 3.7 m high, covered with translucent polyethylene of 0.15 mm thickness, to control temperature and prevent bees from naturally pollinating flowers. In 2000 and 2001, a total of 8 and 6 trees were enclosed, respectively. Electric heating and cooling units were placed in each enclosure to establish two different temperature ranges. Half of the trees were subjected to a high temperature range (11-28° C or average of 21.5° C in 2000; 9-32° C or average of 21.9° C in 2001) and the other half to a low temperature range (8-25° C or average of 13.4° C in 2000; 7-28° C or average of 14.1° C in 2001).

When blossoms at the "popcorn" stage appeared in peak numbers, all opened flowers on designated branches were removed. The next day, all unopened flowers were removed. The remaining flowers, all of which had opened within 24 h, were used in the experiment. On the first day flowers were open, half of the flowers on each tree were pollinated with a mixture of 'Rome' and 'Red Delicious' apple pollen using a paint brush, and the other half were left unpollinated. Flowers were sampled daily (or every other day from trees in low-temperature enclosures in 2001), inoculated in the laboratory with strain Ea153, E325 or A506 using a suspension of 10^8 cfu/ml, incubated for 24 h at 24° C as described for crab apple flowers, then population size determined. Five pollinated and five non-pollinated flowers were collected per strain per tree on each sampling date. Additional flowers (five pollinated and five non-pollinated) were collected on each date and placed in a fixative solution for later examination with a scanning electron microscope.

Stigma Aging and Microbial Interactions

The effect of temperature, pollination and flower stigma age on the interaction between antagonist and the pathogen was studied using crab apple flowers. Flowers were collected and pollinated or not pollinated as described previously. Flowers were held for 0, 1, 2, 3, 4, 5, 6, 8 or 10 days at 14 or 24° C prior to inoculation with antagonist strain E325, C9-1 or A506 using a suspension of 10^8 cfu/ml. (All flowers were inoculated on the same date.) Flowers were then incubated at 24° C for 24 h prior to inoculation with pathogen strain Ea153 using a suspension of 10^7 cfu/ml. After another 24 h of incubation at 24° C, population size of both antagonist and pathogen on each flower was determined. Five pollinated and five non-pollinated flowers were used per stigma age and temperature. Data from two trials were pooled.

RESULTS AND DISCUSSION

When flower stigmas were inoculated with strain Ea153 and flowers incubated at constant or fluctuating temperatures averaging 14° C, the bacterial population increased from about 25 cfu to nearly 10^6 cfu per flower in a 72h period. After 24, 48 and 72 h, the population size on stigmas was not different according to analysis of variance (ANOVA) and the least significant difference test ($P \leq 0.05$). This result seemed to validate the use of constant temperatures in the range of 4 to 24° C in later experiments.

The temperature range at which bacterial populations were shown to increase on

flower stigmas (by exceeding the starting population of 10^4 cfu/ml) varied depending on strain. Based on nonlinear regression curves, the approximate range for Ea153, E325, C9-1 and A506 was 9 to 39° C, 8 to beyond 40° C, 9 to beyond 40° C, and 11 to 34° C, respectively. The optimal temperature for growth of strains Ea153, E325, C9-1 and A506 on stigmas was approximately 27°, 28°, 32° and 25° C, respectively. The results were not unexpected given previous reports based on growth of these bacterial species or strains on artificial media.

The temperature range of strain A506 was inside the range of the pathogen and strains of *P. agglomerans*. Strain A506 is often combined with strain C9-1 (Johnson et al., 1993) or another strain of *P. agglomerans* (Vanneste and Yu, 1996) in biological control studies. The temperature range of such mixtures is likely only as wide as that of *P. agglomerans* alone. Strains E325 and C9-1 grew on stigmas in the full temperature range of *E. amylovora* and at temperatures above the range of the pathogen.

The time period that stigmas of crab apple flowers had the capacity to be colonized when inoculated and to support bacterial growth (indicated by a population size exceeding the starting population of 10^4 cfu per flower) decreased as temperature increased, and it was shorter when flowers were pollinated versus not pollinated. Interactions involving stigma age, temperature and pollination were highly significant ($P < 0.001$) in experiments with detached crab apple flowers according to ANOVA.

These relationships were confirmed in trials with orchard trees of Gala apple in 2000 and 2001. According to quadratic curves fitted to the data, the duration that non-pollinated stigmas in low-temperature enclosures had the capacity to support bacterial growth when sampled and inoculated in the lab was about 12 days for strain A506 and 14 days for strains Ea153 and E325. Non-pollinated stigmas in high-temperature enclosures retained the capacity to support growth of A506, E325 and Ea153 for about 5, 6 and 7 days, respectively. Pollinated stigmas in low-temperature enclosures retained the capacity to support growth of A506 for about 7 days, but supported growth of Ea153 and E325 for up to 12 days. Pollinated stigmas in high-temperature enclosures had the capacity to support growth of A506 for only 3 or 4 days and growth of Ea153 and E325 for 5 or 6 days. The experiment revealed that flower stigmas are conducive to bacterial colonization and growth for longer periods than previously thought (Gouk and Thomson, 1999), depending on temperature and pollination. It also showed that strain A506 failed to multiply at late stages of stigma senescence, which did allow growth of strain E325 and the pathogen.

Flowers sampled from 'Gala' apple for scanning electron microscopy are still being evaluated. However, in general, the collapse and degradation of the stigma papillae over time appears to correspond to a decrease in stigma conduciveness to bacterial colonization and growth as reported previously (Gouk and Thomson, 1999).

When stigma age, temperature and pollination were studied in relation to microbial interactions on crab apple stigmas, antagonist strains were generally shown to reduce populations of *E. amylovora* strain Ea153 throughout the period when stigmas were conducive to colonization and growth by the pathogen. The antagonist strains differed in their capacity to exclude or suppress pathogen populations and these differences were consistent during the period when stigmas were conducive to pathogen growth. Strain E325 was most effective, followed by strain C9-1, then strain A506.

Information generated in these studies has relevance not only to biological control of fire blight, but possibly also to disease prediction modeling.

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Literature Cited

Gouk, S.C. and Thomson, S.V. 1999. Influence of age of apple flowers on growth of *Erwinia amylovora*. Acta Hort. 489:525-528.

- Ishimaru, C. and Klos, E.J. 1984. New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* 74:1342-1345.
- Johnson, C.G. 1940. The maintenance of high atmospheric humidities for entomological work with glycerol-water mixtures. *Ann. Appl. Biol.* 27:295-297.
- Johnson, K.B., Stockwell, V.O., McLaughlin, R.J., Sugar, D., Loper, J.E. and Roberts, R.G. 1993. Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology* 83:995-1002.
- Johnson, K.B. and Stockwell, V.O. 1998. Management of fire blight: A case study in microbial ecology. *Ann. Rev. Phytopathol.* 36:227-248.
- Lindow, S.E, McGourty, G. and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. *Phytopathology* 86:841-848.
- Pusey, P.L. 1997. Crab apple blossoms as a model for research on biological control of fire blight. *Phytopathology* 87:1096-1102.
- Pusey, P.L. 1999. Control of fire blight on pome fruit trees with *Erwinia herbicola*. United States Patent No. 5,919,446.
- Soltész, M., Nyeki, J. and Szabo, Z. 1996. Receptivity of sexual organs. Pages 132-152 in: *Floral Biology of Temperate Zone Fruit Trees and Small Fruits*. J. Neki and M. Soltész, eds. Akademiai Kiado, Budapest, 1996.
- Vanneste, J.L. and Yu, J. 1996. Biological control of fire blight using *Erwinia herbicola* Eh252 and *Pseudomonas fluorescens* A506 separately or in combination. *Acta Hort.* 411:351-353.